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RAPID COMMUNICATION

Ascorbic acid increases the yield of dopaminergic neurons derived from basic fibroblast growth factor expanded mesencephalic precursors

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Abstract

CNS precursors derived from E12 rat mesencephalon proliferate in the presence of basic fibroblast growth factor and differentiate *in vitro* into functional dopaminergic neurons, which upon transplantation alleviate behavioral symptoms in a rat model of Parkinson's disease. Here we show that the efficiency of dopaminergic differentiation decreases in the mesencephalic precursors that were proliferated or passaged for extended periods *in vitro*. Ascorbic acid treatment restored dopaminergic differentiation in these precursors and led to a greater than 10-fold increase in dopamine neuron yield compared with untreated cultures. The effect of ascorbic acid was stereospecific and could not be mimicked by any other antioxidants. The expression of sodium-dependent vitamin C transporter, a recently identified stereospecific ascorbic acid transporter, was maintained in mesencephalic precursors for extended *in vitro* periods. Pre-treatment of *in vitro* expanded mesencephalic precursors with ascorbic acid might facilitate the large-scale generation of dopaminergic neurons for clinical transplantation.

Keywords: midbrain, progenitor cell, stem cells, tyrosine-hydroxylase.

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Recent *in vivo* studies and work in explant cultures defined a number of genes that are critically involved in dopamine (DA) neuron development such as fibroblast growth factor 8, sonic hedgehog (SHH) and Nurr1 (Zetterström *et al.* 1997; Ye *et al.* 1998). A great effort has been devoted to utilizing these factors for the *in vitro* DA conversion of CNS precursor cells. However, all these studies were of very limited success with the possible exception of Nurr1 transfection, which, in combination with yet undefined factors, induced DA features in a stem-like cell line (Wagner *et al.* 1999). We recently described a system that allows the *in vitro* proliferation of ventral mesencephalic precursors. Upon mitogen withdrawal these expanded precursors efficiently differentiate into functional DA neurons that restore behavioral abnormalities in a rat model of Parkinson's disease (Studer *et al.* 1998a). The system yields an expansion factor of 10 over one passage. In absolute numbers, a total number of DA neurons equivalent to that found in seven adult rat brains can be obtained from a single embryonic mesencephalon. However, here we report that the long-term proliferation of mesencephalic precursors *in vitro* leads to a decreased efficiency of DA neuron generation, despite continued neuronal differentiation and survival. We have attempted various strategies to restore DA differentiation. The work presented here is based on a screening approach of a wide-range of factors including neurotrophins, morphogens, cytokines, cyclic nucleotides, cell adhesion factors, neurotransmitters and anti-oxidants towards a goal of improving dopaminergic differentiation in long-term precursors.

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Abbreviations used: AA, Ascorbic acid; BDNF, brain-derived neurotrophic factor; bFGF, basic fibroblast growth factor; CMF-HBSS, Ca^{2+}/Mg^{2+}-free Hanks balanced salt solution; DA, dopamine; DAC, D-acetyl cysteine; dbcAMP, dibutyryl cyclic adenosine monophosphate; DIV, days *in vitro*; EGF, epidermal growth factor; GDNF, glial cell line-derived neurotrophic factor; GSH, glutathione; GSNO, *S*-nitroso-glutathione; iso-AA, D-isoascorbic acid; NT, neurotrophin; P0,1,2,3, passage 1,2,3; PDGF, platelet-derived growth factor; rpHPLC, reverse-phase HPLC; SHH, sonic hedgehog; SVCT, sodium-dependent vitamin C transporter; TH, tyrosine-hydroxylase; TUJ1, β-tubulin type III.

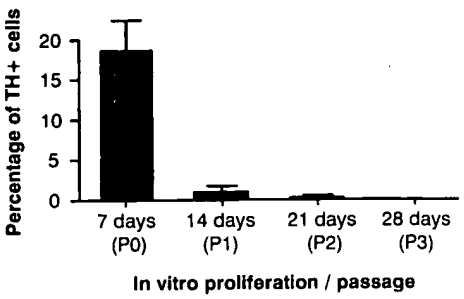

Fig. 1 Decreasing DA yield in long-term expanded mesencephalic precursors. The percentage of TH+ cells in the total cell population was assessed 5 days after differentiation. Precursors were expanded with bFGF for 7, 14, 21 and 28 days *in vitro* and passaged every 7 days (P0, P1, P2, P3). Data represent the mean ± SEM of three independent experiments.

Among all factors tested, ascorbic acid (AA) treatment of long-term basic fibroblast growth factor (bFGF) expanded mesencephalic precursors increased the yield of DA neurons by more than 10-fold. The effect of AA on CNS precursors was of special interest in light of the recent description of a specific transporter that leads to intracellular AA concentrations as high as 5 mM in neurons (Tsukaguchi *et al.* 1999). This result suggests novel roles for AA in DA neuron development, and presents a simple yet effective strategy for improving the efficiency of DA neuron generation for transplantation in Parkinson's disease.

Materials and methods

Culture of mesencephalic precursors

All animal experiments were carried out according to NIH guidelines. Timed-pregnant Sprague–Dawley rats were purchased from Taconic farms. The ventral midbrain was dissected from embryonic rats on embryonic day 12.5 (E12.5; plug day = 0.5). Single-cell suspension was obtained by mechanical trituration in Ca^{2+}/Mg^{2+}-free Hank's balanced salt solution (CMF-HBSS). Cells were plated on 10-cm culture dishes and maintained in serum-free medium in the presence of 20 ng/mL bFGF (Studer *et al.* 1998a). The cultures were passaged after 6–7 days *in vitro* (DIV) at ~90% confluence by incubation in CMF-HBSS for 45–60 min at room temperature, and mechanical removal of the cells with a cell scraper. Twenty-five μL of a cell suspension (8×10^5 cells/mL) were plated into each well in 24-well culture plates (Costar) pre-coated with polyornithine/fibronectin. Circular glass coverslips (12 mm in diameter, Carolina Biologicals) were used to grow cultures subjected to immunofluorescence analysis. After passaging, cells were expanded for an additional 4–5 days in bFGF, with subsequent differentiation for 5 days in the absence of the mitogen. Cells grown for RT-PCR analyzes were serially passaged onto 10-cm plates (1.2×10^6 cells/plate). One 10-cm plate was lyzed at each passage after 6–7 days of bFGF treatment.

Immunohistochemical staining

Standard immunohistochemical procedures were performed. Samples were incubated in the primary antibodies overnight at 4°C. The following primary antibodies were used. (1) Mouse monoclonal anti-tyrosine hydroxylase (anti-TH) 1 : 1000 (Sigma); antiβ-tubulin type-III (clone TUJ1) 1 : 750 (BabCo). (2) Polyclonal: rabbit anti-TH 1 : 200–1 : 500 (PelFreeze); rabbit anti-GABA 1 : 2000 (Sigma); rabbit antiglutamate 1 : 500 (Sigma); rabbit antiaromatic acid decarboxylase 1 : 100 (Protos Biotech Corp); rabbit antidopamine-β-hydroxylase 1 : 100 (Protos Biotech Corp); rabbit antiserotonin (5-HT) 1 : 5000 (Sigma). For quantitative studies appropriate biotinylated secondary antibodies were used, followed by amplification with the Vectastain Elite kit (Vector Laboratories) and visualization with a metal-enhanced 3,3-diaminobenzidine substrate (Pierce). Fluorescence double immunohistochemistry was carried out using appropriate Cy2 and Cy3 tagged secondary antibodies (Jackson ImmunoResearch) or Cy2-streptavidin upon application of biotinylated secondary antibodies. Glass coverslips were dehydrated and mounted with Permount (Fisher Scientific).

Reverse-phase HPLC determination of DA content

Liquid samples were stabilized with 1 : 20 dilution of 0.44% sodium bisulfite (in 7.5% phosphoric acid) and stored at −80°C. DA was extracted by aluminum adsorption. Conditions, settings and equipment utilized for reverse-phase HPLC (rpHPLC) have been described previously (Studer *et al.* 1996; Studer *et al.* 1998a). Results were normalized against DA standards (Chromosystems) and confirmed by co-elution at varying flow rates and oxidative potentials.

RT-PCR

Cultures were washed in phosphate buffered saline once before solubilization in Trizol (Gibco Life Technologies) then stored at −80°C. RNA extraction was carried out according to the manufacturer's recommendations. Superscript kit (Gibco Life Technologies) was used for reverse transcription of 10 μg RNA per condition. The annealing temperatures for all the primers were calculated (MAC-Vector) and PCR conditions were optimized by varying $MgCl_2$ concentration and cycle number in order to determine the linear amplification range. Amplification products were identified by size and confirmed by DNA sequencing. $MgCl_2$ concentrations for TH were 2 mM, and for all others 1.5 mM. Glyceraldehyde-3-phosphate dehydrogenase and TH PCR amplification were carried out as described previously (Studer *et al.* 2000). Primer sequences, cycle numbers, annealing temperatures and product sizes for the other primers were: SVCT1 – f.CCAGATTCAGCAGGGACTTCCACC, b.CCGGATGCCCACTGTAGTCTGAATG, 35 cycles, 61°C, 285 bp; SVCT2 – f.AGCTGCAGGCAGGTGATAAGCG; b.CTTGGCCTCCTCTTCATATTTGCC, 35 cycles, 61°C, 237 bp.

RT-PCR analyzes were confirmed in two independent culture series.

Quantification and statistical analysis

Immunoreactive cells (TH, serotonin, TUJ1) were counted in 10 uniform randomly chosen areas of each well using an eye piece grid at a final magnification of 200 or 320. Three–six culture wells were analyzed in each experiment. Data are expressed as mean ± SEM from three independent culture series. ANOVA was carried out followed by post hoc comparisons [Newman–Keuls (multiple groups), Dunnett (compared with control group)].

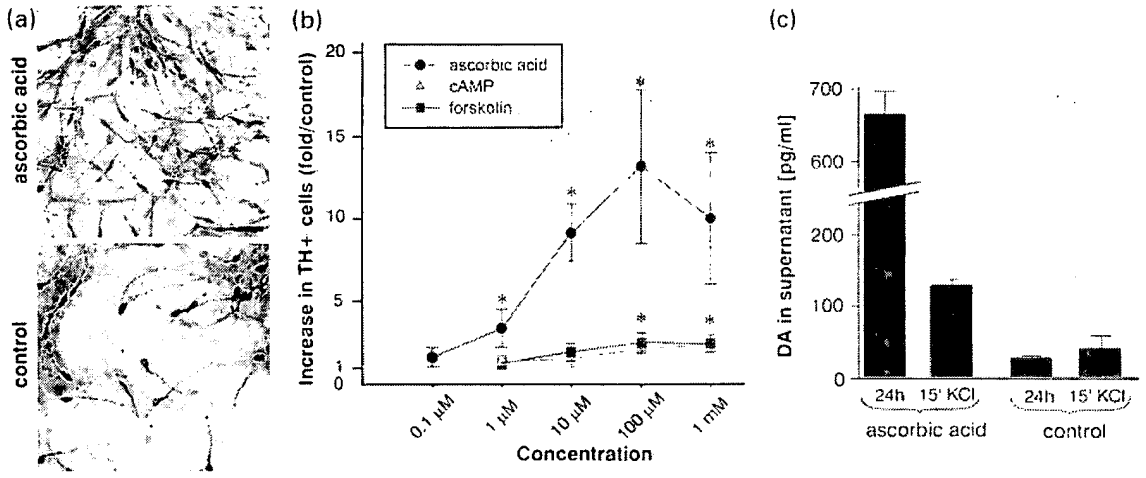

Fig. 2 AA increases generation of DA neurons in passaged (P1) mesencephalic precursors. (a) Cells treated with 100 µM AA during 5 days of cell differentiation showed a marked increase in TH+ cells compared with control cultures (scale bar = 20 µm). (b) Dose dependent increase in TH yield was observed for AA, dbcAMP and forskolin. Data represent the mean ± SEM of three independent experiments ($^*p < 0.05$). (c) AA mediated increase in TH+ cells was reflected by rpHPLC analyzes showing increased DA levels in the conditioned medium and after KCl-evoked release. Data represent the mean ± SEM of four samples from one representative experiment ($n = 4$).

Results

The yield of TH + cells derived from mesencephalic precursors decreases after subculture

The efficient DA differentiation of short-term expanded precursors was lost after long-term expansion and passaging (Fig. 1). In bFGF-proliferated, but unpassaged precursors 18.0 ± 3.6% of the total cell population were TH+ cells [passage 0 (P0) cells; average expansion rate 9-fold]. In cells passaged after 7 days of bFGF proliferation and subsequently expanded for another 7 days (P1 − 80-fold expanded) the percentage of TH+ cells upon mitogen withdrawal dropped to 1.2 ± 0.9% ($p < 0.01$). Continued passaging and proliferation further reduced the percentage of cells expressing TH, and after three passages (P3 − 5400-fold expanded) the TH cell yield was < 0.01%. In contrast to the dramatic decrease in TH+ cells there was a much smaller loss of the TUJ1+ total neuronal population over passaging (P0 = 63 ± 12%; P1 = 33 ± 13%; P2 = 23 ± 11%; P3 = 17 ± 10%) indicating that DA neuron differentiation is specifically affected.

AA and cAMP agonists increase the yield of TH + cells in mesencephalic precursors after subculture

We tested an extensive list of reagents for their ability to restore dopaminergic differentiation in passage 1 (P1) mesencephalic precursors. The P1 stage was chosen because the loss in DA differentiation was most dramatic between the P0 and P1 stages (Fig. 1). All factors applied during P1 proliferation (in the presence of bFGF), such as FGF1, FGF4, FGF8, epidermal growth factor (EGF), brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF) and platelet-derived growth factor (PDGF) did not affect the TH yield. The following factors were tested during the P1 differentiation phase (in the absence of bFGF when precursor cells differentiate):

neurotrophins and cytokines − BDNF, neurotrophin-3 (NT3),

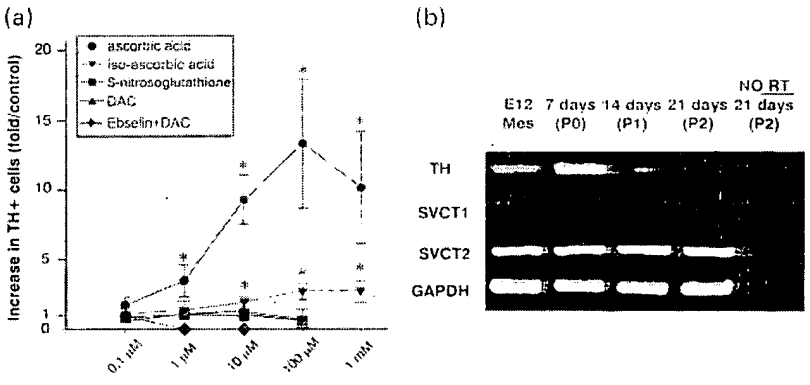

Fig. 3 Alternative antioxidants do not mimic the AA effect on TH yield. (a) Iso-AA is the only alternative agent with a significant effect on the TH yield, but it is orders of magnitude less effective than AA ($^*p < 0.05$). (b) RT-PCR analysis for AA transporter in CNS precursor cells: Increased *in vitro* proliferation led to decreased TH expression by RT-PCR. The type 2 AA transporter (SVCT2) was expressed in all precursors whereas no SVCT1 mRNA could be detected. Control samples were treated identically but in the absence of reverse transcriptase (RT) to exclude contamination with genomic DNA.

NT4/5, GDNF, neurturin, interleukin-1α and FGF1; morphogens – FGF8, SHH alone or in combination with FGF8; neurotransmitters – GABA and DA; cyclic nucleotides/agonists – dibutyryl cyclic AMP (dbcAMP), cGMP and forskolin;

Extracellular matrix proteins – laminin, collagen V, heparan-sulfate, entactin-collagen-laminin;

Antioxidants and cofactors – tetrahydrobiopterin, AA, glutathione (GSH), *S*-nitrosoglutathione (GSNO), ebselin (an agent mimicking GSH-peroxidase), α-tocopherol (Vitamin E) and *N*-acetyl-L-cysteine.

A significant twofold increase in TH+ cells derived from P1 precursors was observed with dbcAMP or forskolin treatment during cell differentiation ($p < 0.05$, Fig. 2b). Treatment with 100 μM AA during the whole differentiation period led to a greater than 10-fold increase in TH+ cells ($p < 0.05$, Figs 2a and b). AA added during days 1–2 after mitogen withdrawal (6.5-fold increase in TH+ cells) was more effective than during days 3–5 (4-fold increase). AA treatment during P1 cell proliferation did not increase the TH yield. AA also increased the number of precursor-derived serotonin neurons, another ventral neuronal subtype (AA 1.8% ± 0.4% versus control 0.2% ± 0.4%, $p < 0.05$). There was no change in the total number of neurons in cultures treated with AA (31 ± 8% TUJ1+ in AA versus 33 ± 13% in the control).

TH+ cells generated in the presence of AA are functional DA neurons

During development and under specific culture conditions several different cell types such as GABAergic striatal cells can express TH, thus not all TH+ cells are DA neurons (Max *et al.* 1996; Daadi and Weiss 1999). Immunohistochemical analyzes showed that TH+ cells in our study were positive for aromatic acid decarboxylase, but negative for dopamine-β-hydroxylase and GABA. Additional confirmation of a functional DA phenotype was obtained by measuring cumulative DA levels (24-h period in culture medium), and evoked DA release (15 min in buffer solution with 56 mM KCl) by rpHPLC. Both total DA content and KCl-evoked DA release were increased in cultures treated with AA (Fig. 2c).

The effect of AA on dopaminergic differentiation cannot be mimicked with other antioxidants or increased DA levels.

AA function is often based on its antioxidative properties. However, none of the other potent antioxidants tested, such as reduced GSH, α-tocopherol (vitamin E), s-nitrosoglutathione (GSNO), N-acetyl-L-cysteine and Ebselin (a GSH-peroxidase analog) could mimic the AA effect on the TH yield (Fig. 3a). The stereoisomer D-iso-ascorbic acid (iso-AA) significantly increased TH+ cell numbers but was much less effective than AA. Iso-AA has the same antioxidative properties as AA, but is not actively transported into the cells via the stereospecific transporters SVCT1 and SVCT2. We tested whether SVCT1 or SVCT2 are expressed in our precursor cells using RT-PCR analysis (Fig. 3b). In agreement with the histochemical data, precursor cells expressing TH mRNA were drastically reduced after long-term culture. SVCT2 expression was found in both short-term and long-term proliferated precursors, whereas no expression of SVCT1 could be detected. These findings suggest that AA treatment increases DA neuron yield via an intracellular mechanism, and demonstrate expression of the stereospecific AA transporter SVCT2 in long-term proliferated midbrain precursor cells.

Discussion

In vitro bFGF expansion of mesencephalic precursors is a promising tool to overcome some of the practical and ethical concerns of fetal tissue transplantation in Parkinson's disease. Current technology allows the expansion of TH+ cell number as well as enrichment in the percentage of TH+ cells grafted (Studer *et al.* 1998a). Further improvements in precursor cell technology are geared towards increasing the efficiency of DA neuron generation from long-term expanded precursors and towards improving *in vivo* cell survival (Brundin and Bjorklund 1998; Studer *et al.* 1998b). Our screen for candidate extrinsic factors revealed that simple supplementation of the medium with AA and cAMP agonists can greatly enhance the yield of precursor derived DA neurons. Beyond the practical implications for clinical transplantation, the unexpected effect of AA on mesencephalic precursors might provide novel insights into DA neuron differentiation. AA is best known for its role as an essential nutrient (vitamin C) in guinea pigs and primates. AA deficiency in these species leads to a pathological condition known as scurvy. AA also has important roles as a water-soluble antioxidant and serves as cofactor in many biochemical reactions including catecholamine synthesis (Nakashima *et al.* 1970). Furthermore, it enhances iron absorption and red blood cell formation and promotes osteogenic differentiation in mesenchymal stem cells (Jaiswal *et al.* 1997).

Antioxidative agents including AA have been shown to improve the survival of mesencephalic DA neurons in primary cell culture (Kalir and Mytilineou 1991). Such agents are generally most effective in mature DA neurons probably because of their ability to counteract the toxic effects of endogenous DA (Rosenberg 1988). AA treatment in our study was more effective during days 1–2 of differentiation than during days 3–5, indicating that AA affected an early step of DA neuron differentiation. Immunohistochemistry for 8-hydroxyguanine and antinitro tyrosine, two proteins commonly used to identify oxidative damage in DNA/RNA (Helbock *et al.* 1999) and proteins (Pennathur *et al.* 1999), did not show any differences between AA treated and control cultures (data not shown). Furthermore, treatment with alternative antioxidative agents was unable to mimic the AA effect. In summary, our data suggest a novel role for AA on DA neuron differentiation independent of its antioxidative properties.

Another potential mechanism for increased numbers of DA neurons could be increased extracellular DA levels in AA treated cultures as AA inhibits DA autooxidation. This possibility appeared attractive as DA exposure *in vitro* is known to increase the number of TH+ cells in primary cortical and striatal cultures (Max *et al.* 1996; Zhou *et al.* 1996). However, DA treatment in the presence of AA levels sufficient to prevent auto-oxidation, but insufficient to mimic the full effect of AA on DA differentiation, did not lead to increased TH yield (data not shown). While eliminating a few possible modes of action, our current study does not reveal the mechanisms by which AA affects TH-yield in CNS precursors. AA treatment of various other cell types has been implicated in the direct or indirect activation of tissue-specific promoters such as collagen (for a review see (Ronchetti *et al.* 1996) or osteocalcin

promoters (Xiao *et al.* 1997). AA might similarly activate cell differentiation pathways in CNS precursor cells that affect the DA neuron yield.

Several interesting observations have been recently made about the presence of AA in the CNS (Rice 2000). The brain has the highest AA levels of any organ system because of the presence of a specific AA-transporter system in neurons that maintains the steep intra/extracellular concentration gradient. AA concentrations are especially high in forebrain structures including the neostriatum, which is rich in DA terminals. Evidence suggests that the substantia nigra is the critical site controlling AA release in the neostriatum (Rebec and Pierce 1994). Glutamate neurons and glial cells exhibit AA release coupled with glutamate uptake and both DA and glutamatergic transmission are modulated in a complex manner by AA. The presence of SVCT2 on CNS precursors demonstrated here suggests that these cells might be useful as an *in vitro* assay system for further defining the role of AA in the CNS. AA treatment promoted differentiation of DA and serotonergic neurons. These two neuron types develop in the ventral CNS and can both be induced by SHH and FGF8 in neural plate explants (Ye *et al.* 1998). Although SHH and FGF8 had no significant effect on TH yield in our system, future studies must address whether genes downstream of SHH and FGF8 might contribute to the specification of ventral neuron fates promoted by AA. Our data demonstrate a powerful effect of AA on the number of DA neurons generated from bFGF-expanded mesencephalic precursor cells. The findings contribute towards efficient *ex-vivo* generation of DA neurons for clinical transplantation and raise interesting questions about the role of AA in brain development.

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Ref rences

Brundin P. and Bjorklund A. (1998) Survival of expanded dopaminergic precursors is critical for clinical trials. *Nature Neurosci.* **1**, 537–537.

Daadi M. M. and Weiss S. (1999) Generation of tyrosine hydroxylase-producing neurons from precursors of the embryonic and adult forebrain. *J. Neurosci.* **19**, 4484–4497.

Helbock H. J., Beckman K. B. and Ames B. N. (1999) 8-Hydroxydeoxyguanosine and 8-hydroxyguanine as biomarkers of oxidative DNA damage. *Methods Enzymol.* **300**, 156–166.

Jaiswal N., Haynesworth S. E., Caplan A. I. and Bruder S. P. (1997) Osteogenic differentiation of purified, culture-expanded human mesenchymal stem cells in vitro. *J. Cell Biochem.* **64**, 295–312.

Kalir H. H. and Mytilineou C. (1991) Ascorbic-acid in mesencephalic cultures – effects on dopaminergic neuron development. *J. Neurochem.* **57**, 458–464.

Max S. R., Bossio A. and Iacovitti L. (1996) Co-expression of tyrosine hydroxylase and glutamic acid decarboxylase in dopamine differentiation factor-treated striatal neurons in culture. *Dev. Brain Res.* **91**, 140–142.

Nakashima Y., Suzue R., Sanada H. and Kawada S. (1970) Effect of ascorbic acid on hydroxylase activity. I. Stimulation of tyrosine hydroxylase and tryptophan-5-hydroxylase activities by ascorbic acid. *J. Vitaminol. (Kyoto)* **16**, 276–280.

Pennathur S., Jackson-Lewis V., Przedborski S. and Heinecke J. W. (1999) Mass spectrometric quantification of 3-nitrotyrosine, ortho-tyrosine, and o,o′-dityrosine in brain tissue of 1-methyl-4-phenyl-1,2,3, 6-tetrahydropyridine-treated mice, a model of oxidative stress in Parkinson's. *J. Biol. Chem.* **274**, 34621–34628.

Rebec G. V. and Pierce R. C. (1994) A vitamin as neuromodulator – ascorbate release into the extracellular fluid of the brain regulates dopaminergic and glutamatergic transmission. *Prog. Neurobiol.* **43**, 537–565.

Rice M. E. (2000) Ascorbate regulation and its neuroprotective role in the brain. *Trends Neurosci.* **23**, 209–216.

Ronchetti I. P., Quaglino D. Jr and Bergamini G. (1996) Ascorbic Acid and Connective Tissue, in *Ascorbic Acid: Biochemistry and Biomedical Cell Biology* (Harris R., ed.), Plenum Press, New York.

Rosenberg P. A. (1988) Catecholamine toxicity in cerebral cortex in dissociated cell culture. *J. Neurosci.* **8**, 2887–2894.

Studer L., Csete M., Lee S.-H., Kabbani N., McKay R. D., Wold B. and McKay R. D. (2000) Enhanced proliferation, survival and dopaminergic differentiation of CNS precursors in lowered oxygen. *J. Neurosci.* **20**, 7377–7383.

Studer L., Psylla M., Bühler B., Evtouchenko L., Vouga C. M., Leenders K. L., Seiler R. W. and Spenger C. (1996) Non-invasive dopamine determination by reversed phase HPLC in the medium of free-floating roller tube cultures of rat fetal ventral mesencephalon. A tool to assess dopaminergic tissue prior to grafting. *Brain Res. Bull.* **41**, 143–150.

Studer L., Tabar V. and McKay R. D. (1998a) Transplantation of expanded mesencephalic precursors leads to recovery in parkinsonian rats. *Nature Neurosci* **1**, 290–295.

Studer L., Tabar V. and McKay R. D. (1998b) Survival of expanded dopaminergic precursors is critical for clinical trials. *Nature Neurosci.* **1**, 537–537.

Tsukaguchi H., Tokui T., Mackenzie B., Berger U. V., Chen X. Z., Wang Y. X., Brubaker R. F. and Hediger M. A. (1999) A family of mammalian Na+-dependent L-ascorbic acid transporters. *Nature* **399**, 70–75.

Wagner J., Akerud P., Castro D. S., Holm P. C., Canals J. M., Snyder E. Y., Perlmann T. and Arenas E. (1999) Induction of a midbrain dopaminergic phenotype in Nurr1- overexpressing neural stem cells by type 1 astrocytes. *Nature Biotechnol.* **17**, 653–659.

Xiao G., Cui Y., Ducy P., Karsenty G. and Franceschi R. T. (1997) Ascorbic acid-dependent activation of the osteocalcin promoter in MC3T3-E1 preosteoblasts: requirement for collagen matrix synthesis and the presence of an intact OSE2 sequence. *Mol Endocrinol.* **11**, 1103–1113.

Ye W. L., Shimamura K., Rubenstein J. R., Hynes M. A. and Rosenthal A. (1998) FGF and Shh signals control dopaminergic and serotonergic cell fate in the anterior neural plate. *Cell* **93**, 755–766.

Zetterström R. H., Solomin L., Jansson L., Hoffer B. J., Olson L. and Perlmann T. (1997) Dopamine neuron agenesis in Nurr1-deficient mice. *Science* **276**, 248–250.

Zhou J. W., Bradford H. F. and Stern G. M. (1996) Induction of dopaminergic neurotransmitter phenotype in rat embryonic cerebrocortex by the synergistic action of neurotrophins and dopamine. *Eur. J. Neurosci.* **8**, 2328–2339.